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The relation between growth phases, cell volume changes and metabolism of adherent cells during cultivation

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ABSTRACT

In biotechnology, mathematical models often consider changes in cell numbers as well as in metabolite conversion to describe different cell growth phases. It has been frequently observed that the cell number is only a delayed indicator of cell growth compared to the biomass, which challenges the principle structure of corresponding models.

Here, we evaluate adherent cell growth phases in terms of cell number and biomass increase on the basis of detailed experimental data of three independent cultivations for Madin Darby canine kidney cells. We develop a model linking cell numbers and mean cell diameters to estimate cell volume changes during growth without the need for diameter distribution measurements. It simultaneously describes the delay between cell number and cell volume increase, cell-specific volume changes and the transition from growth to maintenance metabolism while taking different pre-culture conditions, which affect the cell diameter, into account. In addition, inspection of metabolite uptake and release rates reveals that glucose is mainly used for generation of cellular energy and glutamine is not required for cellular maintenance. Finally, we conclude that changes in cell number, cell diameter and metabolite uptake during cultivation contribute to the understanding of the time course of intracellular metabolites during the cultivation process.

1. Introduction

Adherently growing cells start with a lag phase in which they attach to the new growth surface and initiate proliferation. Exponential growth follows in which the cells spread over the surface until space becomes limiting, which is known as cell density-dependent growth inhibition (Stoker and Rubin, 1967). As cells consume substrates, decreasing metabolite levels may also limit proliferation (e.g. Frame and Hu, 1991; Glacken et al., 1986). Independent of which resource is limiting, when the specific growth rate decreases the cells pass through an intermediate phase to the stationary phase characterized by maintenance metabolism.

A variety of mathematical models have been developed that account for multiple growth inhibiting aspects to study different growth phases of mammalian cells (Bock et al., 2009; Cherry and Papoutsakis, 1989; Dhir et al., 2000; Frame and Hu, 1988; Goudar et al., 2005; Miller et al., 2000; Möhler et al., 2008; Pörtner and Schäfer, 1996). The underlying structure of cell growth models has a mainly empirical form where the biological system is viewed as a catalyst for the conversion of substrates into products (Teixeira et al., 2007; Tziampazis and Sambanis, 1994). To elucidate the substrate demands that arise in each of the growth phases, metabolite uptake rates are typically related to the cell number concentration and its increase to account for maintenance and growth metabolism, respectively.

Experimental studies of Frame and Hu (1990) and Adams (1969) show that cells increase in size well before they divide, resulting in a delay between the biomass increase and cell number increase. Thus, it appears reasonable to interpret the cell number as a delayed indicator of growth (Frame and Hu, 1990; Nielsen et al., 1997; Park et al., 2010). A lag phase in cell number growth is typically implemented, and the corresponding models fail to describe the uptake and release of metabolites experimentally identified during the initial phase of cultivations. Toward the end of cultivations, cell density-dependent growth inhibition can occur and cells enter a stationary growth phase. If cell density-dependent growth inhibition is calculated on the basis of cell numbers, a distorted view on growth phases might result as cell numbers can increase while the biomass remains constant. At the end of cultivation, final cell numbers might be different between cultivations inoculated under the same conditions (e.g. Möhler et al., 2008). Obviously, higher cell concentrations can correlate with smaller mean cell diameters (Erlinger and Saier, 1982). Thus, cell number-based models can fit several cultivations but only in a very restrictive way. It can

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Nomenclature

Latin letters

$\bar{d}$	mean cell diameter (μm)
d_m	minimum diameter (μm)
d_c	critical diameter (μm)
f	growth inhibition factor
$[Glc]$	glucose concentration (mmol L^{-1})
$[Gln]$	glutamine concentration (mmol L^{-1})
k_{dGln}	specific glutamine decomposition rate constant (h^{-1})
F_{evap}	water evaporation rate constant (L h^{-1})
I	identity matrix
K_m	Monod constant (mmol L^{-1})
$[Lac]$	lactate concentration (mmol L^{-1})
M	system matrix
m_{Glc}	cell volume-specific glucose uptake rate for maintenance (mmol L^{-1} μl^{-1} h^{-1})
m_{Gln}	cell volume-specific glutamine uptake rate for maintenance (mmol L^{-1} μl^{-1} h^{-1})
Nc	number of classes
r_{dGln}	glutamine decomposition rate (mmol L^{-1} h^{-1})
$r_{m/Glc}$	medium volume-specific uptake rate of glucose for maintenance (mmol L^{-1} h^{-1})
$r_{m/Gln}$	medium volume-specific uptake rate of glutamine for maintenance (mmol L^{-1} h^{-1})
r_{trans}	transition rate (h^{-1})
$r_{X/Glc}$	medium volume-specific uptake rate of glucose for growth (mmol L^{-1} h^{-1})
$r_{X/Gln}$	medium volume-specific uptake rate of glutamine for growth (mmol L^{-1} h^{-1})
s	adjustable parameter
V^C	cell volume (μl)
V^C_{model}	model cell volume (μl)
V^C_{Si}	volume of cells in class i (μl)
V^C_*	approximate cell volume for larger times (μl)
V^M	medium volume (L)
X_i	number of cells in class i (cells)
X_{tot}	cell number (cells)
$Y_{X/Glc}$	cell growth-specific yield coefficient of glucose (mmol L^{-1} $cells^{-1}$)
$Y_{X/Gln}$	cell growth-specific yield coefficient of glutamine (mmol L^{-1} $cells^{-1}$)
$Y_{Lac/Glc}$	glucose-specific lactate yield

Greek letters

a	scaling factor
λ_k	eigenvalues of M
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
ρ	parameter of system
τ	time delay (h)

be observed that yield coefficients, maintenance constants as well as growth rates show large confidence intervals. Biomass-based models may solve some of these problems but cannot distinguish between cell-specific volume changes and cell number increase. Especially when considering intracellular biochemical processes, parameters can correlate with the cell volume (e.g. protein synthesis, membrane composition) as well as with the cell number (e.g. nucleus activities, signaling pathways). Hence, a model is desired that renders both the cell volume as well as the cell number. Furthermore, cell-specific volume changes are required to estimate intracellular reaction rates and are likewise not provided by simple biomass-based models. Nevertheless, both approaches are simple and, have without question, a certain power in analyzing cultivations of mammalian cells.

To overcome the limitations of existing models, we develop a model that considers cell number, mean cell diameter and cell volume to describe growth of adherent Madin Darby canine kidney (MDCK) cells in six-well plates. In addition, time courses of the extracellular concentrations of glucose, glutamine and lactate as well as of the intracellular concentrations of glucose 6-phosphate were taken into account. Parameters of the model are estimated from three independent experiments with different pre-culture conditions. Triplicate measurements at individual sampling time points enable assessment of model quality. Different growth phases are analyzed as well as the delay between the time courses of cell volume and cell number. Finally, cell-specific metabolite uptake rates are used to examine cellular metabolite demands during different growth phases and to link uptake of glucose to glycolysis.

2. Materials and methods

2.1. Cultivation

Madin Darby Canine Kidney (MDCK) cells (ECACC, #84121903) were pre-cultured in GMEM (Gibco, #22100-093), supplemented with 10% fetal calf serum (Gibco, #10270-106), 2 g L^{-1} peptone (International Diagnostics Group, #MC33) and 4 g L^{-1} $NaHCO_3$ (Roth, #6885.1), referred to as GMEM-Z.

Pre-culture at 37 °C was either carried out in roller bottles (Greiner Bio-One, #680XX, experiment depicted in figures with ○ symbol) or in T-flasks (Greiner Bio-One, #661160, experiments depicted in figures with Δ and □) with 5% CO_2.

The actual experiments were carried out in parallel 6-well plates (Greiner Bio-One, #657160) containing 4 ml GMEM-Z with an initial cell concentration of 0.66×10^6 cells $well^{-1}$ (experiment Δ), 0.45×10^6 cells $well^{-1}$ (experiment ○) and 0.74×10^6 cells $well^{-1}$ (experiment □), cultivated at 37 °C and 5% CO in an incubator.

2.2. Analytics

For each cultivation at least three wells were sacrificed per time point and analyzed separately. After removal of the supernatant, the cells were washed 3 times with PBS (8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 0.2 g L^{-1} KH_2PO_4, 1.2 g L^{-1} Na_2HPO_4) and treated 30 min with porcine trypsin (2.5%) 0.5 mg $well^{-1}$ (Gibco, #27250-018). Cells were harvested using a cell scraper. A Vi-Cell TM XR Cell Viability Analyzer (Beckman Coulter) was used for cell counting and measurement of the mean cell diameter (average of the mean cell diameter determined individually for a total number of 100 images per sample). The cell number and the (overall) mean cell diameter were used to calculate a cell volume of the culture. Error bars of the cell volume were calculated according to the error propagation law. Calculation of the maximum specific growth rate was based on logarithmic transformation.

Concentrations of glucose, glutamine and lactate in the supernatant were quantified by using a Bioprofile 100 plus analyzer (Nova Biomedical, relative standard deviation of the method according to Genzel and Reichl (2007): 1.2–1.6%). Glucose 6-phosphate was quantified by anion-exchange chromatography in combination with mass-spectrometry as described in Ritter et al. (2006) and Ritter et al. (2008) (relative standard deviation of the method according to Ritter et al. (2006) is 2.0%).

2.3. Computation

Model fitting, estimation of the parameter confidence intervals and visualization of the results were programmed in MATLAB©